APPENDIX B

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Effects of CD28 Costimulation on Long-Term Proliferation of CD4$^+$ T Cells in the Absence of Exogenous Feeder Cells[1]

Bruce L. Levine,[2]* Wendy B. Bernstein,† Mark Connors,‡ Nancy Craighead,§ Tullia Lindsten,¶ Craig B. Thompson,¶ and Carl H. June*

In this report, conditions for prolonged in vitro proliferation of polyclonal adult CD4$^+$ T cells via stimulation with immobilized anti-CD3 plus anti-CD28 have been established. CD4$^+$ cells maintained exponential growth for more than 60 days during which a total 10^9- to 10^{11}-fold expansion occurred. Cell cultures exhibited cyclical changes in cell volume, indicating that, in terms of proliferative rate, cells do not have to rest before restimulation. Indeed, electronic cell size analysis was the most reliable method to determine when to restimulate with additional immobilized mAb. The initial ~10^5-fold expansion was autocrine, occurring in the absence of exogenous cytokines or feeder cells. Addition of recombinant human IL-2 after the initial autocrine expansion resulted in continued exponential proliferation. Phorbol ester plus ionomycin also induced long-term growth when combined with anti-CD28 stimulation. Analysis of the T cell repertoire after prolonged expansion revealed a diverse repertoire as assessed by anti-TCR Vβ Abs or a PCR-based assay. Cytokines produced were consistent with maintenance of both Th1 and Th2 phenotypes; however, the mode of CD3 and CD28 stimulation could influence the cytokine secretion pattern. When anti-CD3 and anti-CD28 were immobilized on the same surface, ELISAs on culture supernatants revealed a pattern consistent with Th1 secretion. Northern analysis revealed that cytokine gene expression remained inducible. Spontaneous growth or cell transformation was not observed in more than 100 experiments. Together, these observations may have implications for gene therapy and adoptive immunotherapy. Furthermore, these culture conditions establish a model to study the finite lifespan of mature T lymphocytes. *The Journal of Immunology,* 1997, 159: 5921–5930.

In vivo, T lymphocytes require at least two signals for complete activation. In addition to an Ag signal, a second costimulatory signal allows a T helper cell to produce sufficient IL-2 and other cytokines to allow autocrine-driven clonal expansion (1, 2). The CD28 receptor on T cells is able to provide such a costimulatory signal following interaction with CD80 or CD86 on APCs (2–4). CD28 stimulation has been shown to stabilize cytokine mRNA and can also induce the cell survival gene Bcl-X_l (5). Indeed, the antiapoptotic effect appears to be a major role for CD28 ligation in vivo (6). Recently, the CCR1, CCR2, and CCR5 β chemokine receptors have been identified as the first described genes that are down-regulated following CD28 stimulation (7, 8).

Besides CD28, CD80 and CD86 are also able to bind to another related receptor on T cells, CTLA4, with a higher affinity than for CD28 (9–11). Recent evidence has indicated that the role of CD152 (CTLA4) may be to deliver a negative signal (12) to an activated cell, resulting in anergy or cell death (13), which may be important in clearing cells from the site of an immune response (14). Further, mice deficient of CTLA4 die of massive T cell proliferation within a few weeks of birth (15, 16). Therefore, the relative expression of CD28 or CTLA4 on the surface of a T cell may determine whether the cell will become activated and continue to divide, become tolerant, or die.

The control of T cell homeostasis is complex and not completely understood. The lifespan of normal T cells is heterogeneous, as T cells have been shown to consist of multiple subsets of cells composed of both long-lived and short-lived cells (17). In humans receiving radiotherapy for nonlymphoid malignancies, the intermitotic survival time of naïve T cells is estimated to be about 3.5 years and for memory T cells, about 22 wk (18). To begin to gain an understanding of post-thymic T cell survival, it is necessary to know the replicative capacity of T cells as well as the intermitotic survival time. Because of the ability of CD28 to induce the cell survival gene Bcl-X_l (5), we began studies directed at developing a culture system that would allow the polyclonal proliferation of purified CD4$^+$ T cells. We thus developed a method to stimulate CD4$^+$ T cells that may provide for more optimal CD28 stimulation, with less "feeder cells" expressing B7-1 or B7-2 that might trigger CTLA4. Signal 1 is provided by a mAb directed against CD3, and the costimulatory signal is provided by a mAb directed against CD28 that does not cross-react with CTLA4. In addition, this system allows for long-term polyclonal expansion of HIV-infected CD4 cells (19) due to down-regulation of HIV coreceptors (7). We report that mixtures of adult naïve and memory CD4 cells can undergo about 30 to 40 population doublings (PDs).[3] The cells

*Henry M. Jackson Foundation for the Advancement of Military Medicine, U.S. Military HIV Research Program, Bethesda, MD 20889; †Division of Retrovirology, Walter Reed Army Institute for Research, Rockville, MD 20850; ‡National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; §Immune Cell Biology Program, Naval Medical Research Institute, Bethesda, MD 20889; and ¶Gwen Knapp Center and Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637

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[2] Address correspondence and reprint requests to Dr. Bruce L. Levine, Mail Stop 061, Naval Medical Research Institute, 8901 Wisconsin Ave., Bethesda, MD 20889-5607.

[3] Abbreviations used in this paper: PD, population doubling; PDL, population-doubling level.

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retain a highly diverse TCR repertoire and can be induced to secrete mixtures of cytokines characteristic of Th1 or Th2 cells, depending on the form of CD28 stimulation. This system may be useful in adoptive immunotherapy and gene therapy protocols, not only for viral infections, but also for the treatment of malignancies in which the rapid expansion of purified T cells is desirable.

Materials and Methods

Antibodies

For cell purifications, the following purified and azide-free mAbs were used: anti-CD8 OKT8 (IgG2a), anti-CD11b OKM1 (IgG2b), anti-CD14 63D3 (IgG1), anti-CD16 3G8 (IgG1), anti-CD20 1F5 (IgG2a), and anti-HLA-DR 2.06 (IgG1). All of the hybridomas were obtained from the American Type Culture Collection (Rockville, MD) except 3G8, which was a kind gift of Dr. Stephen Shaw (National Institutes of Health, Bethesda, MD). For stimulations, anti-CD3 OKT3 (IgG1) and anti-CD28 9.3 (IgG2a) were used.

Cells

PBLs were isolated by Percoll gradient centrifugation from leukopacks obtained by apheresis of healthy donors. $CD28^+CD4^+$ T cells were purified as described previously (20) by a negative selection method using magnetic beads (Dynal, Lake Success, NY). In each experiment the purity of the separation was monitored by flow cytometry: $CD28^+$ $CD4^+$ T cells were >98% $CD3^+$, >98% $CD28^+$, and <3% $CD8^+$.

Long-term cell cultures

Cells were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine (BioWhittaker), and 20 mM HEPES (BioWhittaker). OKT3 and 9.3 were bound to magnetic beads (Tosylactivated M-450; Dynal) at ~150 fg per bead (*cis* stimulation). In some experiments, as noted in figure legends, *trans* stimulation was conducted as follows: 1) Dynal beads were coated with anti-CD28 mAb 9.3 or anti-CD3 mAb OKT3, and equal mixtures of anti-CD3 and anti-CD28 beads added to cells; 2) for plastic-immobilized anti-CD3 stimulation, OKT3 was precoated on the culture wells or flask by overnight incubation with a 10 μg/ml solution. The culture wells or flasks were washed extensively with PBS before use and anti-CD28 stimulation performed by addition of 9.3 mAb at 1 μg/ml. Dynal beads were added to T cells at one to three beads per cell with the cells at 1×10^6 cells per/ml in complete medium. The cultures were fed at 2- to 3-day intervals to maintain a concentration of 1 to 2×10^6 cells/ml. Beads were not removed from culture, but were diluted progressively until restimulation. The cell cultures were counted and monitored for cell size or volume on a Coulter Counter model ZM and Channelyzer model 256 (Coulter, Hialeah, FL) equipped with a 70-μ long-bore orifice tube and restimulated with additional anti-CD3/anti-CD28-coated beads when the volume of the T cell blasts decreased to <400 femtoliters. For unstimulated cells, a lower gate was set at 25.5 femtoliters and for activated cells, a lower gate was set at 76.5 femtoliters so that paramagnetic beads would not be counted along with cells. Cell counts were determined from the total particles above these gates, and viability as assayed by trypan blue dye exclusion was routinely >95%. No exogenous cytokines or feeder cells were added to the cultures. Recombinant human IL-2 was added to certain cultures as indicated, but only after the cells did not respond to the addition of fresh anti-CD3/anti-CD28-coated beads by an increase in cell volume several days after the initial stimulation. PD time was determined by analysis of the exponential phase of cell growth by linear regression using statistical functions in SigmaPlot 3.0 and the formula PD in h = 24 × Log2/B1 where B1 = slope of the line number of cells plotted against days.

CDR3 size analysis to determine TCR Vβ diversity

RNA was extracted from 1×10^7 purified $CD4^+$ cells using TRIZOL Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized with the SuperScript Preamplification System for First Strand cDNA Synthesis kit (Life Technologies) according to the manufacturer's instructions using 5 μg of total RNA, oligo(dT) 12-18 (0.5 μg/μl), and Superscript II reverse transcriptase. The reaction was incubated at 42°C for 5 min, reverse transcriptase added with an additional 50-min incubation, and the reaction terminated at 70°C for 15 min. Residual RNA was removed by incubation with RNaseH at 37°C for 20 min.

CDR3 length of the variable β region was determined using PCR amplification originally described by Pannetier (21–23). cDNA from purified $CD4^+$ cells was amplified using Vβ region primers for each of 20 Vβ families and a Cβ region (24). Each PCR consisted of 1× Promega (Madison, WI) 10× buffer, 0.2 mM dNTP, 1.7 mM $MgCl_2$, 0.6 μM Cβ primer, 18 μg cDNA, and 0.5 U *Taq* coupled with TaqStart Ab (Clontech, Palo Alto, CA). The reaction was conducted in a 9600 Perkin-Elmer thermocycler (Norwalk, CT) for 40 cycles: 90°C for 25 s followed by 40 cycles of 90°C for 25 s, 60°C for 45 s, 72°C for 45 s, followed by an extension period at 72°C for 5 min. A 2-μl aliquot of this reaction was then submitted to a one-cycle runoff reaction using a 6-FAM-labeled Cβ primer. The samples were then mixed with deionized formamide, Rox-500 size standard (Perkin-Elmer), and dextran blue and subjected to electrophoresis on a 373 Applied Biosystem Sequencer (Foster City, CA) using a 24-cm well to read plates and an 8 M urea, 6% polyacrylamide gel. Data were then analyzed using the GeneScan Software 672 Analysis Software (Perkin-Elmer).

Cytokine assays

Cytokine contents in long-term cultures were determined upon restimulation by washing the cells in fresh medium, restimulation with beads, and collection of supernatants after 24 h. Concentrations of cytokines in cell-free supernatants were assayed by ELISA using commercially available kits obtained from the following sources: IL-2, T Cell Diagnostics/Endogen (Woburn, MA); IL-4, R&D Systems (Minneapolis, MN) or Dianova (Hamburg, Germany); IL-5, TNF-α, granulocyte macrophage-CSF, MIP-1α, MIP-1β, RANTES, R&D Systems; IL-10, Dianova; IL-13, Biosource (Camarillo, CA); IFN-γ, Endogen (Woburn, MA) or R&D Systems. All values reported were assessed by using dilutions of culture supernatant that yielded read-outs within the linear portion of the standard curve.

Results

CD28 costimulation specifically augments long-term $CD4^+$ T cell proliferation

Previous studies have shown that CD28 costimulation can specifically augment the proliferation of T cells during short-term culture. To more fully characterize the proliferative potential of adult $CD4^+$ T cells, cells were initially incubated with either anti-CD3 + anti-CD28 coimmobilized on beads, PHA + recombinant human IL-2 or anti-CD3 immobilized on beads plus recombinant human IL-2, as shown in Figure 1*A*. In earlier experiments, the ratio of anti-CD3 (OKT3) to anti-CD28 (9.3) conjugated to the beads was titrated and optimal long-term proliferation was observed at a ratio of 1:1 (data not shown). Only cells stimulated with the combination of anti-CD3 and anti-CD28 exhibited long-term growth. Cells cultured with optimal amounts of anti-CD3 plus recombinant human IL-2 or PHA plus recombinant human IL-2 exhibited similar growth rates for the initial 2 to 3 wk of culture compared with anti-CD3 plus anti-CD28-stimulated cells. However, the CD28-costimulated cells remained in exponential proliferation and the anti-CD3 plus recombinant human IL-2 and PHA plus recombinant human IL-2-stimulated cultures entered the plateau phase of growth in the 2nd to 3rd weeks of culture. Cell numbers in the experiment shown in Figure 1*A* had increased ~1 $\log_{10}$ more in cultures stimulated with anti-CD3 plus anti-CD28 than cultures stimulated with anti-CD3 + recombinant human IL-2 or PHA + recombinant human IL-2 by day 20 of culture. The input cells were typically >97% $CD28^+$, >95% $CD4^+$ (data not shown). It is important to note that APC were removed by negative immunomagnetic selection before the initiation of the culture. This was assured by the failure of the $CD4^+$ cells to grow in the presence of PHA alone. Furthermore, the proliferation induced by anti-CD3/CD28-coated beads was entirely autocrine, as exogenous cytokines or feeder cells were not added to the culture. Finally, the ability of anti-CD28 to sustain cell proliferation was specific, as beads coated with anti-CD3 and a variety of other Abs to T cell surface structures such as MHC class I, CD4, CD5, CD7, CD43, CD45, CD40L, and CTLA4 did not exhibit sustained proliferation (25, and data not shown). Thus, increased cell adhesion to the beads cannot account for specific enhancement of growth by CD28.

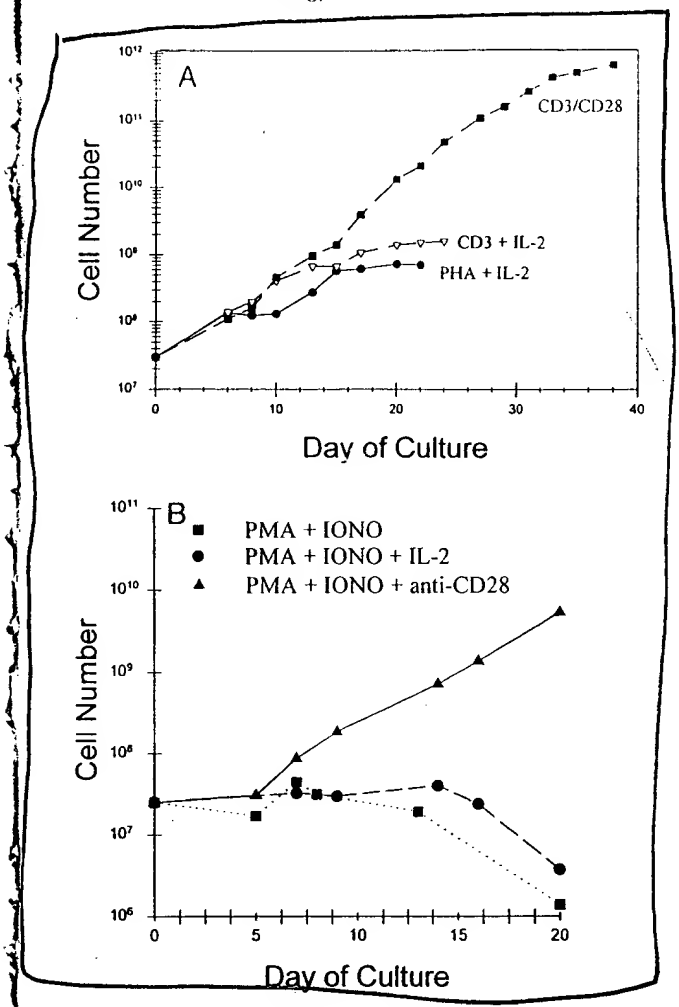

FIGURE 1. CD28 costimulation mediates growth of peripheral blood $CD4^+CD28^+$ T cells in the absence of exogenous cytokines or feeder cells and does not require Ag receptor stimulation. *A*, Anti-CD3 (OKT3) + anti-CD28 (9.3)-coated Dynal beads (solid squares), or anti-CD3 (OKT3)-coated Dynal beads + recombinant human IL-2 100 U/ml (open triangles), or PHA 5 μg/ml + recombinant human IL-2 100 U/ml (solid circles) were added to $CD4^+$ T cells. *B*, PMA 1.9 nM + ionomycin 0.08 μg/ml (solid squares), or PMA 1.9 nM + ionomycin 0.08 μg/ml + recombinant human IL-2 100 U/ml (solid circles), or PMA 1.9 nM + ionomycin 0.08 μg/ml + anti-CD28 (9.3)-coated Dynal beads (solid triangles) were added to $CD4^+$ T cells. Fresh medium was added to the cultures as required and excess cells discarded as described in *Materials and Methods*. Where indicated, recombinant human IL-2 was added to media for cells grown in recombinant human IL-2 to maintain a concentration of 100 U/ml. Cell number was determined using the average of two counts on a Coulter Counter ZM. The total number of cells that would be expected to accumulate is displayed, taking into account discarded cells.

To further delineate the specificity of anti-CD28 to induce long-term autocrine proliferation, $CD4^+$ T cells were stimulated with either PMA plus ionomycin, PMA plus ionomycin plus recombinant human IL-2, or PMA plus ionomycin plus anti-CD28 as shown in Figure 1*B*. Earlier experiments established optimal concentrations of PMA and ionomycin for T cell proliferation (data not shown). The addition of anti-CD28 prolonged and enhanced proliferation observed with PMA + ionomycin. Recombinant human IL-2 could not replace the effect of CD28 addition, similar to the result observed in Figure 1*A* in the context of anti-CD3 stimulation. The inability of recombinant human IL-2 addition to completely replace the growth-promoting effects of CD28 costimulation is likely related to the ability of CD28 and not IL-2 to induce sustained expression of the antiapoptotic gene Bcl-X_l (5). Another possibility is that CD28 may preserve the responsiveness of cells to IL-2 receptor stimulation. Recently, mouse T cell clones first stimulated with Ag and later by cross-linking the TCR were shown to become unresponsive to IL-2 (26). In addition, this experiment indicates that surface engagement of the TCR by anti-CD3 is not required for the growth-potentiating effects of CD28 costimulation, as pharmacologic "bypass" activation appears to be sufficient. Furthermore, a prolonged lag phase was observed in cultures of PMA plus ionomycin plus anti-CD28 stimulation, shown in Figure 1*B*, as revealed by comparing the lag phase in cultures stimulated with anti-CD3 and anti-CD28, shown in Figure 1*A*. The explanation for the increased lag phase in PMA/ionomycin-stimulated cultures is not fully known, but is likely related to the observation that only a subset of cells bearing CD101 responds to anti-CD28 and PMA stimulation, while essentially all $CD28^+$ cells respond when stimulated with anti-CD3 plus anti-CD28 (27). Together, these experiments confirm and extend previous results showing that CD28 delivers a signal that costimulates T cells stimulated by anti-CD3 or by phorbol esters plus calcium ionophore and results in autocrine proliferation for about 1 mo (28).

Cyclical restimulation of $CD4^+$ T cells with immobilized anti-CD3/CD28 results in long-term exponential growth

In the absence of exogenous recombinant human IL-2 addition we have previously shown that $CD4^+$ T cells stimulated with anti-CD3/CD28 will exhibit exponential growth for about 20 days (28), while $CD4^+CD45RA^+$ (naïve) T cells will exhibit exponential growth for about 45 days (29). In addition, we found that repeated addition of beads coated with anti-CD3/CD28 was necessary to sustain cell proliferation, and that addition of exogenous recombinant human IL-2 could further sustain proliferation. In Figure 2*A*, the growth curve of $CD4^+$ T cells cyclically stimulated by anti-CD3/CD28 beads, as described in *Materials and Methods*, is shown. After the fifth restimulation, the cells are no longer able to maintain logarithmic growth and plateau at 8 $\log_{10}$ above the input number of cells, corresponding to a mean population-doubling level (PDL) of 27. In contrast, the period of exponential growth could be extended to >60 days when recombinant human IL-2 was added to the culture. In the experiment shown in Figure 2*A*, the exponential growth phase was extended to a 2×10^{10}-fold increase, or a PDL of 34, with the addition of exogenous recombinant human IL-2 on day 49 of culture, at a point when the cells had become unresponsive to repeated CD3/CD28 stimulation alone. The phenotype of the cells remained >99% TCR-$\alpha\beta^+CD4^+$ (data not shown). The diversity of the TCR expressed in the cells is shown below.

Summarized in Table I are durations of culture and fold expansion of 10 independent $CD4^+$ T cell cultures performed as described in *Materials and Methods*. The cells could be periodically restimulated with anti-CD3/CD28 beads with or without the addition of exogenous recombinant human IL-2, which was added when the culture was unresponsive to further restimulation with anti-CD3/CD28 alone. In the absence of recombinant human IL-2 addition, the cells had an average 2×10^5-fold expansion and an average duration of culture of 39 days. Long-term CD4 proliferation could be obtained with the combination of CD3/CD28 and recombinant human IL-2 stimulation of CD4 cells, with an average duration of proliferation of 82 days. The proliferation was exponential in all cultures as indicated by the semilog plot of cell number vs days of culture. In the cultures grown in the absence of exogenous recombinant human IL-2, the average PD time was

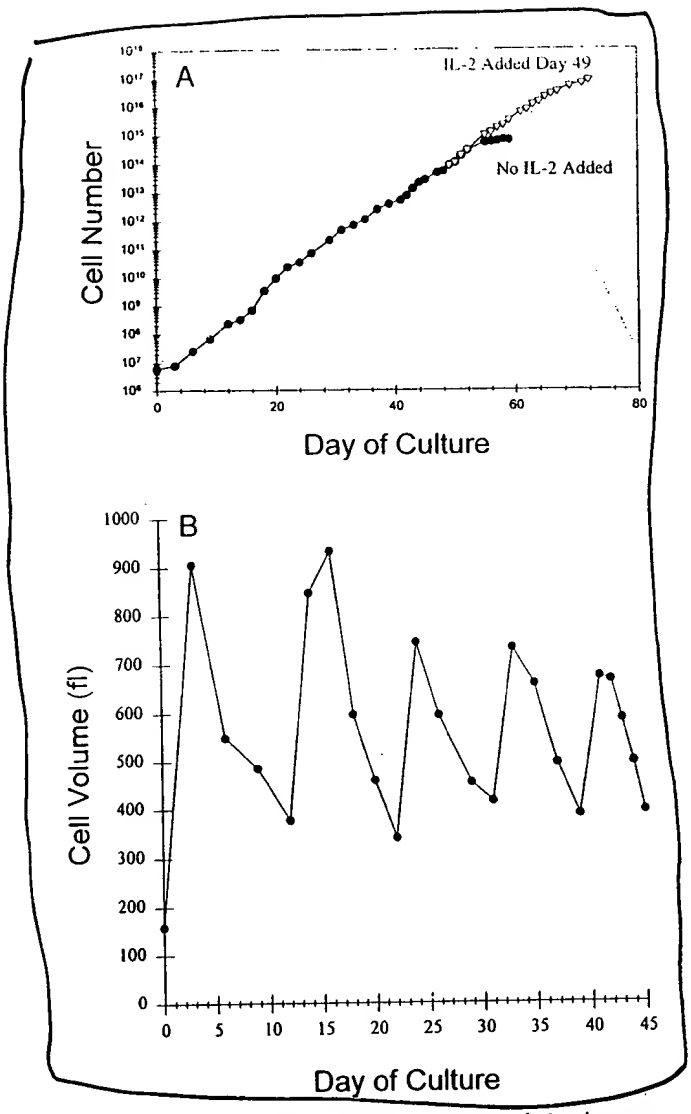

FIGURE 2. Cyclic changes in cell volume occur during long-term exponential proliferation of CD4$^+$ T cells mediated by stimulation with anti-CD3 plus anti-CD28 immobilized on beads. *A*, Growth of CD4$^+$ T cells following six cycles of bead restimulation without the addition of exogenous cytokines (closed circles); the same experiment with the exception of the addition of exogenous recombinant human IL-2 on day 49 (closed circles followed by open triangles). *B*, Mean cell volume (femtoliters) of CD4$^+$ T cells cyclically restimulated with anti-CD3 + anti-CD28-coated beads. At the initiation of the culture, peripheral blood CD4$^+$ T cells had a mean cell volume of 159 femtoliters. Cells were restimulated on days 12, 22, 31, 39, and 45 when the mean cell volume fell below 400 femtoliters. Volume was measured on a Coulter Channelyzer Model C256. For resting day 0 cells, a gate was set to exclude particles <65 femtoliters; for activated cells, a gate was set to exclude particles <200 femtoliters to ensure that beads in the culture were not sized.

37.9 h. In contrast, during the IL-2-dependent phase of cell expansion, the PD time was somewhat slower at 53.2 h. The average PDL for adult CD4$^+$ T cell cultures with exogenous recombinant human IL-2 added was 33 (1×10^{10}-fold expansion). The highest PDL obtained following anti-CD3/CD28 stimulation + recombinant human IL-2 of CD4$^+$ naïve T cells was 41.6 (data not shown). Thus, CD3/CD28 stimulation allows for substantial autocrine-driven proliferation followed by a period of proliferation that is paracrine and dependent upon the addition of exogenous cytokines.

Cyclical restimulation of CD4$^+$ T cells with immobilized anti-CD3/CD28 results in cyclical changes in cell volume independent of proliferative rate

The above results established that exponential proliferation could be maintained for many weeks by periodic restimulation of CD4 cells with anti-CD3/CD28-coated beads. As mentioned earlier, we observed that prolonged exponential proliferation could be achieved if cells were periodically restimulated with Ab-coated beads when cells approached resting cell volumes. In Figure 2*B* the cell volumes are displayed for the growth curve shown in Figure 2*A*. Resting T cells have a mean cell volume of ~170 femtoliters. CD4$^+$ T cells stimulated with immobilized anti-CD3/CD28 increased in volume from 170 femtoliters to nearly 900 femtoliters by 3 days. The cell volume gradually declined over the course of 12 days to 377 femtoliters. At this point, the cells were restimulated by the addition of fresh anti-CD3/CD28 beads and the cell volume again returned to near 900 femtoliters. The culture shown in Figure 2 was restimulated five times, on culture days 12, 22, 31, 39, and 45 before these cells became unresponsive to further restimulation by the addition of anti-CD3/CD28 beads. By comparing Figure 2, *A* and *B*, it is apparent that cyclic changes in cell volume occur that are independent of the rate of cell proliferation. It has been commonly thought that T cell clones need to be "rested" before restimulation. Our results indicate that plateaus in the growth curve are not necessary for cells to regain responsiveness to restimulation. However, a decrease in cell volume appears to serve as an indication that the cells have become responsive to further restimulation. Using this cell stimulation protocol, we have been unable to detect a change in the growth rate of the cells that is related to the readdition of Ab-coated beads. Thus we have concluded that CD4 cell proliferation kinetics can remain exponential and independent of the periodic changes in cell volume. A trivial explanation for the periodic changes in cell volume would be that the beads were included in the cell sizing process, as particles bound to the cell. The beads have a volume that is similar to that of resting cells, and the beads can be electronically gated out from analysis of activated, but not resting, cells. The following observation indicates that the addition of beads does not account for the periodic changes in cell volume, as the beads could be removed from the cell culture on day 3 of culture by magnetic separation, and the beadfree cells were still found to exhibit the cell enlargement and gradual return to near resting volume. Finally, if cells were allowed to grow in culture until their size returned to resting cell volumes, they became unresponsive to restimulation, and cell loss from apoptosis began to occur (data not shown).

Stimulation with immobilized anti-CD3/CD28 of CD4$^+$ T cells results in polyclonal proliferation

To determine whether the cell growth induced by immobilized anti-CD3/CD28 was polyclonal, 10 mAbs directed at 7 different TCR Vβ families covering about 40% of the Vβ repertoire were used to stain cells at the beginning and at day 71 of the culture. As shown in Figure 3, anti-CD3/CD28 stimulation was able to maintain a polyclonal population of CD4$^+$ T cells for 71 days, as all tested Vβ families remained present and there was no variation by more than twofold in the abundance of any given family. The CD4$^+$ T cells shown in Figure 3 are from the same culture shown in Figure 2*B*, so that after 71 days these cells had a mean PDL of 33.2.

Table I. *Summary of proliferative rates of adult CD4⁺ T cells stimulated by immobilized anti-CD3 plus anti-CD28 for 10 separate experiments*

Exp.	Medium	Duration of Culture (days)	Fold Expansion	Population Doubling Time (hours)	Exponential Growth (days)	Correlation Coefficient
1	No IL-2	33	1.8 E + 04	44	14	0.991
2	No IL-2	34	3.2 E + 05	35	17	0.980
3	No IL-2	40	8.3 E + 04	42	19	0.996
4	No IL-2	44	2.3 E + 05	35	16	0.988
5	No IL-2	44	5.0 E + 05	33	16	0.982
Average		39	2.3 E + 05	38	16	0.987
6	IL-2 Added Day 33	65	8.5 E + 05	59	27	0.995
7	IL-2 Added Day 28	76	5.0 E + 09	41	45	0.994
8	IL-2 Added Day 49	78	4.6 E + 10	48	61	0.996
9	IL-2 Added Day 31	84	4.4 E + 08	64	69	0.997
10	IL-2 Added Day 41	106	4.1 E + 08	54	33	0.993
Average		82	1.0 E + 10	53	47	0.995

Cells were stimulated in the absence of feeder cells as indicated in *Materials and Methods*, and for experiments 1–5 cells were grown for the duration of the culture without the addition of exogenous cytokines. For experiments 6–10 cells were grown without the addition of exogneous cytokines until recombinant human IL-2 was added on the indicated day, as exemplified in Figure 2*A*. The proliferative capacity was not assessed in experiments 1–5 as the cultures were terminated while cells remained in exponential growth, whereas experiments 6–10 were terminated when cell cultures began to display a plateau phase. Population doubling (PD) time was determined by analysis of the exponential phase of cell growth by linear regression as described in *Materials and Methods*. The cultures had exponential growth as indicated by the goodness of fit of the growth curve with a straight line. The duration of exponential proliferation is indicated.

To further analyze the effect of prolonged CD4 cell culture on the TCR repertoire, a PCR approach based upon measurement of the length of CDR3 was used. CD4 cells were cultured for 60 days in the presence of anti-CD3 and CD28-coated beads and the TCR clonotypes of 20 Vβ families determined by RNA PCR analysis (22, 30). The PCR products were analyzed on an automated DNA sequencer and the intensity of the bands depicted on plots with the heights corresponding to the intensity of the fluorescent bands. The Vβ repertoire as assessed at the start of culture was diverse as each Vβ family had a gaussian distribution pattern of an average of eight peaks separated by three nucleotides (Fig. 4). Visual inspection after 60 days of culture indicated that a less heterogeneous profile of CDR3 sizes was present. About 75% of the Vβ families remained with diverse profiles while the remaining showed clonal peaks that suggested oligoclonal expansions. Thus, prolonged culture of CD4 cells with anti-CD3 and CD28 can result in the clonal contraction or expansion that is not apparent with FACS analysis. However, this high resolution analysis indicates that the culture retains much of the input diversity.

Cytokine mRNA and protein is more abundant in CD4⁺ T cells stimulated with anti CD3 + anti-CD28 than with anti-CD3 + recombinant human IL-2

It has previously been shown that CD28 stimulation results in increased cytokine production through stabilization of cytokine mRNA as well as through increased de novo synthesis (31). Figure 5 shows that the induction of IL-2 and TNF-α mRNA following anti-CD3/CD28 stimulation is much more pronounced and prolonged than mRNA induced following anti-CD3 + recombinant human IL-2 stimulation. The enhanced cytokine expression did not reflect differences in growth rate, as the cell proliferation was equivalent for the first several weeks of culture, as shown in Figure 1. Cytokine gene expression was not constitutive, as mRNA for IL-2 and TNF was not detected on day 8 before restimulation. However, inducible cytokine mRNA expression was observed on restimulation. To determine whether this increase in cytokine mRNA correlated with secretion, supernatants from CD4⁺ T cells stimulated with anti-CD3 beads + recombinant human IL-2 or anti-CD3/CD28 beads were collected after 24 h and assayed for a variety of cytokines and chemokines by ELISA, as shown in Figure 6. Anti-CD3/CD28 not only induced higher levels of most cytokines by significant amounts, but also induced the secretion of detectable levels of IL-4 and IL-13. The pattern of lymphokine production reflected what has been seen in Th0/Th1 cells (32, 33). Shown in Table II is the level of cytokine contained in supernatants from anti-CD3/CD28-stimulated cells from the CD4 cells shown in Figures 2 and 3. Supernatants were collected 24 h after the first four stimulations with anti-CD3/CD28 beads and corresponding to the day of culture shown in the table. Levels of IL-2 and IFN-γ induced remained high compared with IL-4, indicating the maintenance of a Th0/Th1 phenotype. Earlier studies have shown that long-term culture of T cells with anti-CD3 and anti-CD28 can lead to a population of cells that secretes predominantly Th2-type cytokines (34). However, these studies were performed with anti-CD3 and anti-CD28 in *trans*, that is, with anti-CD3 immobilized on plastic and soluble anti-CD28. If anti-CD3 and anti-CD28 are presented in *cis* (immobilized on the same bead), the present results suggest that a progressive bias toward Th2 cytokine patterns does not occur. These results suggest that the manner in which CD3 and CD28 are stimulated on the surface of T cells results in differential signal strengths or in differential signals generated through the TCR and CD28 that can elicit distinct patterns of cytokine secretion. These findings may prove useful in adoptive immunotherapy approaches where skewing toward a particular cytokine profile is desirable.

Discussion

Conditions have been developed that permit extensive in vitro CD4 cell propagation. Although we are not aware of previous studies documenting long-term (>4 wk) proliferation of polyclonal CD4 cells, anti-CD3 plus anti-CD28 mAbs have been used by others to clone T cells with high efficiency. Riddell and Greenberg (35) cloned CMV-specific CD8⁺ T cells and maintained these cells for 3 mo in culture, at which time they maintained their Ag-lytic activity. Another study has demonstrated cloning of CD4⁺CD45RO⁻ T cells under feeder cellfree conditions. However, the addition of exogenous cytokines was required (36). CD2 stimulation has also been utilized in concert with CD28 stimulation to induce long-term autocrine proliferation of CD4⁺ T cell clones (37).

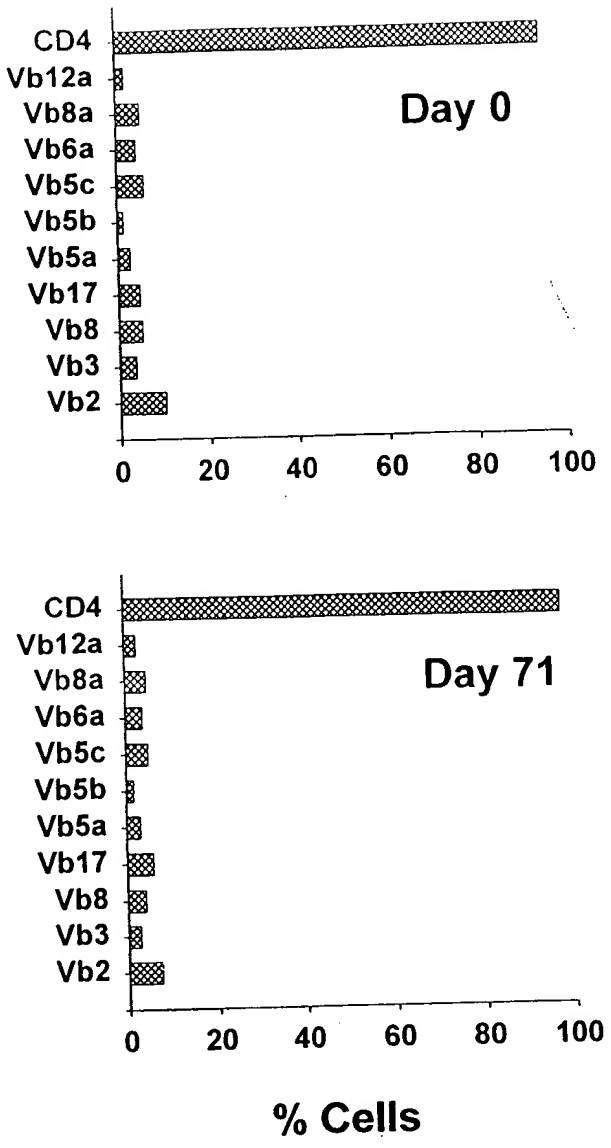

FIGURE 3. Assessment of TCR Vβ repertoire by FACS after anti-CD3- and anti-CD28-mediated proliferation. Polyclonality was assessed by FACS analysis using mAbs to TCR Vβ families with 10 different mAbs (T Cell Diagnostics and Immunotech) covering 7 different TCR Vβ families as well as an anti-CD4 mAb. Cells from the experiment shown in Figure 2 were stained on the day of initiation of the culture (day 0) and after 71 days in culture. Day 71 of the culture corresponds to a 10 $\log_{10}$-fold expansion or 33.2 mean PDs. The percent positive cells was designated as those with fluorescence intensities greater than 98% of cells stained with phycoerythrin- or FITC-conjugated isotype-specific normal mouse Ig as control. Analysis was performed on a Coulter Epics Elite.

Long-term growth of $CD4^+$ T cells following CD3/CD28 stimulation followed a predictable pattern. Exponential growth was observed throughout the culture. In spite of this the cells exhibited periodic changes in cell volume. The basis for the cyclic changes in cell size remain unknown but may relate to differences of cell cycle distribution. Alternatively, the changes in cell size may indicate a previously unrecognized cell growth function that is regulated by CD3/CD28 stimulation. In support of this, we have noted that cells stimulated in costimulation-deficient conditions, such as with mitogenic lectins and recombinant human IL-2, proliferate at equivalent rates and maintain smaller cell volumes. Cell sizing is useful for determining when to restimulate the cultures, thereby avoiding the plateau in growth curves that occurs with standard culture techniques. This is one factor that is important in avoiding apoptosis and maintaining TCR repertoire during prolonged in vitro culture.

Early in the cultures, cells secreted large amounts of IL-2 and other cytokines, allowing for autocrine proliferation in the absence of added cytokines. However, cells eventually required the addition of exogenous recombinant human IL-2 to continue proliferating. Whether this progression from an autocrine to a paracrine state is a natural state of differentiation of peripheral blood-derived $CD4^+$ T cells or a reflection of the in vitro aging induced by this method of culture is currently under investigation. We have observed that CD28 receptor levels decline when $CD4^+$ T cells were cultured for several weeks (data not shown). The absence of CD28 on the cell surface would mean that a costimulatory signal could not be delivered by anti-CD3/CD28-coated beads and thus would account for the necessity of adding exogenous recombinant human IL-2. In fact, the decline in CD28 expression with age (38) and during progression to AIDS (39–42) is well documented. Following the induction of anergy in either resting or activated T cells, CD28 is down-regulated at the level of mRNA (43). Recently Lloyd et al. (44) showed that IL-4 could down-modulate CD28 expression on $CD8^+$ T cells. These $CD28^-$ cells were found to be less responsive to anti-CD3-mediated proliferation than $CD28^+CD8^+$ T cells.

Immobilizing anti-CD3 and anti-CD28 on beads allowed the titration of the signal delivered through the TCR/CD3 complex with the signal delivered through the CD28 receptor. We found that soluble anti-CD28 did not support long-term proliferation equivalent to immobilized anti-CD28. Preliminary studies suggest that this is due to the ability of immobilized anti-CD28 to maintain CD28 expression, whereas soluble CD28 leads to a more rapid loss of CD28 expression (data not shown). The fact that earlier studies have used soluble anti-CD28 may be the primary reason that long-term polyclonal proliferation of CD4 cells has not previously been reported. Timing, or the delivery, of the signal to the TCR as well as accessory molecules can affect cellular responses such as proliferation and apoptosis (45, 46). For this reason we titrated the relative amounts of anti-CD3 (OKT3) and anti-CD28 (9.3) on beads and found that for long-term growth of $CD4^+$ T cells, a ratio of 1:1 was optimal (data not shown). Similarly we determined the optimal ratio of beads to cells for long-term growth and found that there was no difference between 3 beads per cell and 1 bead per cell, but at 0.3 beads per cell long-term growth was not sustained (data not shown). This signal was independent of CD3 ligation per se, as pharmacologic simulation of CD3 stimulation by phorbol ester and calcium ionophore was also enhanced by the addition of anti-CD28 mAb.

CD28 stimulation delivered a specific signal that enhanced cell growth compared with stimulation with anti-CD3 alone. The initial rates of proliferation were equivalent in CD3 and recombinant human IL-2-stimulated cultures compared with CD3 and CD28-stimulated cultures. However, cells stimulated with optimal amounts of anti-CD3 in the presence of exogenous recombinant human IL-2 did not maintain prolonged growth. These results are in agreement with previous studies in the mouse indicating that Ag-dependent clonal expansion of $CD4^+$ T cells in vivo is dependent on CD28 costimulation (47). This may be due to the ability of CD28 to promote lymphocyte survival in both human and mouse T cells (5, 6).

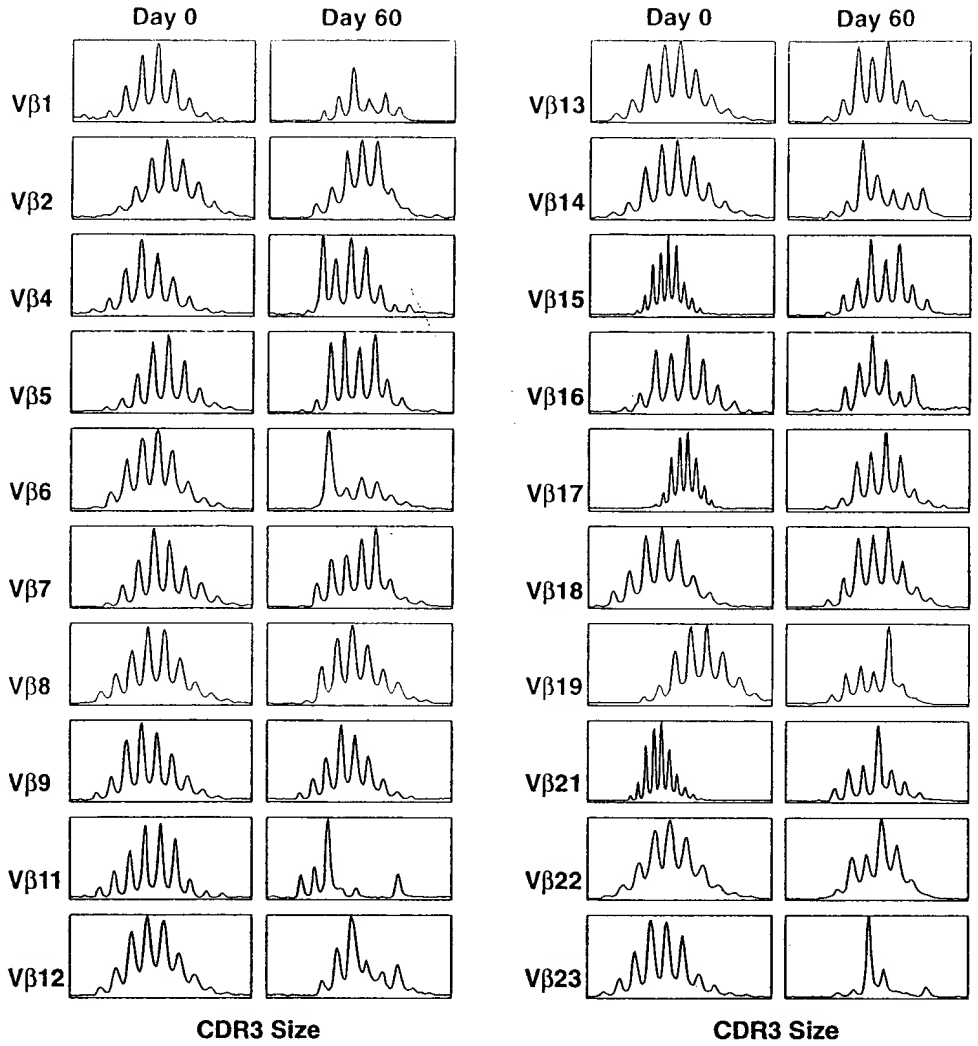

FIGURE 4. Assessment of TCR Vβ repertoire by PCR after anti-CD3- and anti-CD28-mediated proliferation. Polyclonality was assessed by CDR3 length of the variable β region for 20 Vβ families and a Cβ, as described in *Materials and Methods*. Resting $CD4^+$ T cells isolated from an apheresis product (day 0) and CD3/CD28-stimulated cells on day 60 of culture were analyzed. A gaussian distribution of the eight CDR3 length fragments indicates a polyclonal population, and a nongaussian distribution indicates either an expansion or deletions in particular Vβ subsets within the same family.

The ability to propagate Ag-nonspecific or polyclonal T cell lines first became possible following the identification and characterization of IL-2 as a T cell growth factor (48). However, mixed populations of $CD4^+$ and $CD8^+$ would eventually result in a population of cells that was predominantly $CD8^+$ (49). Furthermore, polyclonal T cell propagation has not been demonstrated with IL-2, possibly due to the ability of IL-2 to prime activated T cells for apoptosis. Subsequent studies of the long-term growth of human T cells have necessitated the use of IL-2 in addition to mitogenic lectins and autologous or irradiated allogeneic feeder cells (37, 50, 51).

Our present studies indicate that polyclonal populations of adult CD4 cells can proliferate for a 10^9- to 10^{11}-fold expansion, equivalent to 30 to 40 PDs. Previous studies by Effros and coworkers have addressed the lifespan of T lymphocytes in vitro (50). They reported a mean PD of 23 ± 7 from adult T cells using lectins and feeder cells for cell propagation. We have consistently achieved higher PDLs. Furthermore, while some reports indicate that rare T cells can grow in vitro for up to 80 PDs (52, 53), our present results indicate that this does not appear to reflect the replicative capacity of the vast majority of polyclonal peripheral T lymphocytes. Thus, our studies indicate that the replicative capacity of CD4 cells is extensive but finite. In separate studies we found heterogeneous replicative properties of CD4 cells as we found that naïve cells had a greater replicative capacity than memory CD4 cells (29). In vitro, costimulation by anti-CD28 appears to have a role in telomerase induction (54); however, it remains to be established whether this has any function in determining the long-term proliferative capacity of the cultures that we observe in vitro or in determining T cell replicative capacity in vivo.

The role of CD28 costimulation in T cell differentiation remains controversial. In mice, CD28 costimulation is required to prime Th1 and Th2 cells; however, CD28 appears to promote the differentiation of cells that secrete Th2 cytokines (55). We have examined cytokine production in cultures following stimulation with beads bound with both anti-CD3 and anti-CD28 (*cis* stimulation) and found a maintenance of a Th1 profile of cytokine production over several rounds of stimulation during

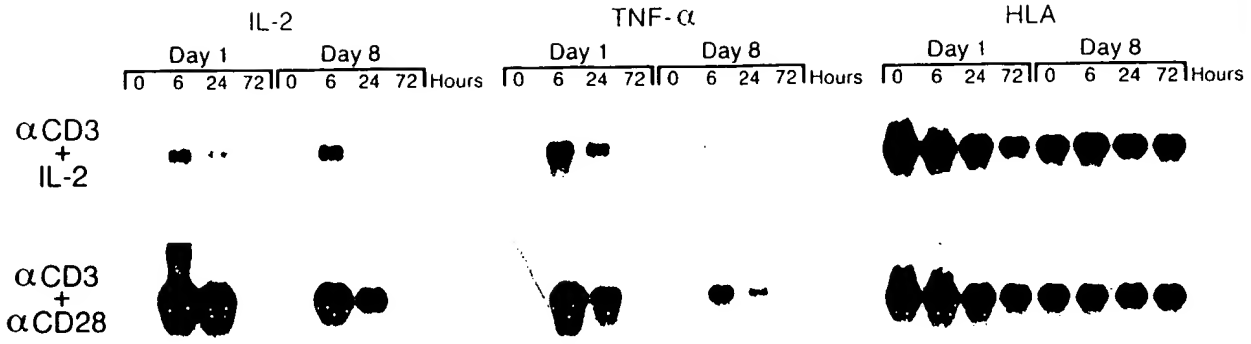

FIGURE 5. Cytokine gene expression remains inducible in cultures of exponentially proliferating $CD4^+$ T cells after anti-CD3 plus anti-CD28 stimulation. $CD4^+CD28^+$ T cells were stimulated and maintained in culture as described in *Materials and Methods*. On day 1 or 8 of the culture, cells were collected and subjected to Northern analysis at 0, 6, 24, and 72 h following stimulation with either anti-CD3 + recombinant human IL-2 100 U/ml or plastic-immobilized anti-CD3 plus anti CD28 1 μg/ml for IL-2, TNF-α, and HLA class I mRNA expression as described in *Materials and Methods*.

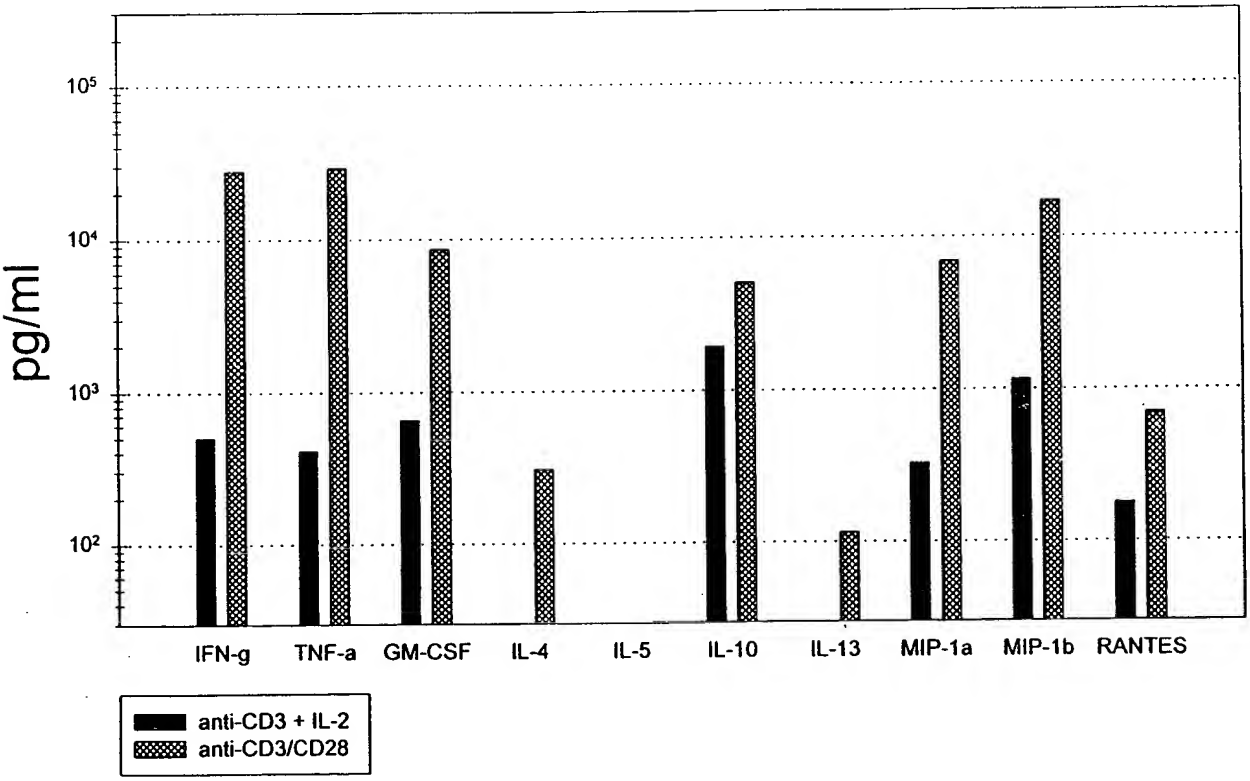

FIGURE 6. Cytokine and β chemokine secretion in cultures of $CD4^+$ cells as measured by ELISA. Anti-CD3- plus anti-CD28-coated beads or anti-CD3-coated beads plus recombinant human IL-2 100 U/ml were added to freshly isolated peripheral blood $CD4^+CD28^+$ T cells at a ratio of three beads per cell. IL-2 as measured by ELISA was 189,600 pg/ml for anti-CD3- plus anti-CD28-coated beads and 40,812 pg/ml for anti-CD3-coated beads plus recombinant human IL-2 100 U/ml. Supernatants for cytokine analysis were collected 24 h later and appropriate dilutions analyzed by ELISA as described in *Materials and Methods*. Sensitivity of the ELISA kits were as follows: IFN-γ, 15.6 pg/ml; TNF-α, 15.6 pg/ml; granulocyte macrophage-CSF, 15.6 pg/ml; IL-4, 15.6 pg/ml; IL-5, 15.6 pg/ml; IL-10, 15.6 pg/ml; IL-13, 19.5 pg/ml; MIP-1α, 31.25 pg/ml; MIP-1β, 31.25 pg/ml; and RANTES, 31.25 pg/ml.

long-term culture. This confirms previous studies that costimulation in *cis* is more efficient than in *trans* (56). Although the mechanism leading to these results remains unknown, we favor the notion that different strengths of Ag dose and costimulation can lead to distinct Th1 and Th2 differentiation. It is also possible that these different forms of stimulation lead to selective survival of Th1- or Th2-like cells, and further studies will be required to distinguish these possibilities. Together, the present results help clarify apparent differences from previous studies of cytokine secretion patterns after CD28 stimulation where repetitive *trans* stimulation could lead to the emergence of a Th2 phenotype (34), while repetitive *cis* stimulation maintained cytokine secretion patterns consistent with a Th1 phenotype (19, 28). Regardless of the mechanism involved, it is possible that these results might be useful to produce polyclonal populations of T cells that have Th1 or Th2 bias.

Table II. *Cytokine secretion from CD4⁺ T cells after four cycles of re-stimulation with immobilized anti-CD3 plus anti-CD28*

Stimulation	Day of Culture	IL-2	IFN-γ	IL-4
1	1	18,875	2,083	<62.5
2	13	40,682	17,917	173
3	23	19,277	14,229	267
4	32	10,099	6,993	<62.5

Supernatants were collected 24 h after each stimulation. For stimulations 2, 3, and 4, cells were washed out of conditioned media and reseeded into fresh media in order to measure de novo cytokine production. Values shown are in pg/ml. Sensitivity of the ELISA kits were as follows: IL-2, 61 pg/ml; IFN-γ, 40 pg/ml; IL-4, 62.5 pg/ml.

Many lines of evidence point to an impairment of the cellular immune system with increasing age. Engwerda, Handwerger, and Fox (57) have shown that the response of both CD4 and CD8 or naïve and memory cells to CD28 costimulation is impaired in aged mice. However, in aged humans there is a decline in CD28 expression (38), and a recent study has detected clonal expansions within certain TCR Vβ subsets in aged humans (58). One reason for decreased immune function or repertoire in the elderly is thought to be related to the decrease in thymic export of T cells following involution. In a study of CD4⁺ T cell regeneration following chemotherapy, Mackall et al. (59) found that age or thymic volume correlated with the ability and speed of CD4 cell counts to return to pretreatment levels. Thus, the ability of peripheral T cells to expand ex vivo and reconstitute an impaired immune system has remained in doubt. The data presented here provide further evidence that substantial ex vivo expansion of polyclonal CD4⁺ T cells is possible following cyclical stimulation with immobilized anti-CD3 and anti-CD28 mAbs. Highly diverse populations of CD4 cells could be maintained for at least 8 wk in cultures. All results are compatible with the notion that this approach leads to physiologic cell growth. Furthermore, in experiments involving cells from more than 100 donors, we have not observed transformation after CD3 and CD28 stimulation. Thus, this approach should permit adoptive immunotherapy and gene therapy strategies for immunodeficiencies and malignancies, as well as facilitate further studies on the replicative capacity of T cells.

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References

1. Bretscher, P. 1992. The two-signal model of lymphocyte activation twenty-one years later. *Immunol. Today 13:74.*
2. June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today 15:321.*
3. Jenkins, M. K., and J. G. Johnson. 1993. Molecules involved in T-cell costimulation. *Curr. Opin. Immunol. 5:361.*
4. Schwartz, R. H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell 71: 1065.*
5. Boise, L. H., A. J. Minn, M. A. Accavitti, C. H. June, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by inducing the expression of Bcl-X_L. *Immunity 3:87.*
6. Sperling, A. I., J. A. Auger, B. D. Ehst, I. C. Rulifson, C. B. Thompson, and J. A. Bluestone. 1996. CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. *J. Immunol. 157:3909.*
7. Carroll, R. G., J. L. Riley, B. L. Levine, Y. Feng, S. Kaushal, D. W. Ritchey, W. Bernstein, O. S. Weislow, C. R. Brown, E. A. Berger, C. H. June, and D. C. St. Louis. 1997. Differential regulation of HIV-1 fusion cofactor expression by CD28 costimulation of CD4⁺ T cells. *Science 276:273.*
8. Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med. 184:569.*
9. Linsley, P. S., J. L. Greene, W. Brady, J. Bajorath, J. A. Ledbetter, and R. Peach. 1994. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity 1:793.*
10. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity 1:405.*
11. van der Merwe, P. A., D. L. Bodian, S. Daenke, P. Linsley, and S. J. Davis. 1997. CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J. Exp. Med. 185:393.*
12. Krummel, M. F., and J. P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J. Exp. Med. 183:2533.*
13. Perez, V. L., L. Van Parijs, A. Biuckians, X. X. Zheng, T. B. Strom, and A. K. Abbas. 1997. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity 6:411.*
14. Bluestone, J. A. 1997. Is CTLA-4 a master switch for peripheral T cell tolerance? *J. Imnunol. 158:1989.*
15. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science 270:985.*
16. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity 3:541.*
17. Tough, D. F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med. 179:1127.*
18. McLean, A. R., and C. A. Michie. 1995. In vivo estimates of division and death rates of human T lymphocytes. *Proc. Natl. Acad. Sci. USA 92:3707.*
19. Levine, B. L., J. D. Mosca, J. L. Riley, R. G. Carroll, M. T. Vahey, L. L. Jagodzinski, K. F. Wagner, D. L. Mayers, D. S. Burke, O. S. Weislow, D. C. St. Louis, and C. H. June. 1996. Antiviral effect and ex vivo CD4⁺ T cell proliferation in HIV-positive patients as a result of CD28 costimulation. *Science 272:1939.*
20. June, C. H., J. A. Ledbetter, M. M. Gillespie, T. Lindsten, and C. B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell Biol. 7:4472.*
21. Cochet, M., C. Pannetier, A. Regnault, S. Darche, C. Leclerc, and P. Kourilsky. 1992. Molecular detection and in vivo analysis of the specific T cell response to a protein antigen. *Eur. J. Immunol. 22:2639.*
22. Pannetier, C., M. Cochet, S. Darche, A. Casrouge, M. Zoller, and P. Kourilsky. 1993. The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments. *Proc. Natl. Acad. Sci. USA 90:4319.*
23. Pannetier, C., J. Even, and P. Kourilsky. 1995. T-cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol. Today 16:176.*
24. Genevee, C., A. Diu, J. Nierat, A. Caignard, P. Y. Dietrich, L. Ferradini, S. Roman-Roman, F. Triebel, and T. Hercend. 1992. An experimentally validated panel of subfamily-specific oligonucleotide primers (V alpha 1-w29/V beta 1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. *Eur. J. Immunol. 22:1261.*
25. Riley, J. L., R. G. Carroll, B. L. Levine, W. Bernstein, D. C. St. Louis, O. S. Weislow, and C. H. June. 1997. Intrinsic resistance to T cell infection with HIV type 1 induced by CD28 costimulation. *J. Immunol. 158:5545.*
26. Miyatake, S., M. Sakuma, and T. Saito. 1997. Induction of interleukin-2 unresponsiveness and down-regulation of the JAK-STAT system upon activation through the T cell receptor *Eur. J. Immunol. 27:1816.*
27. Gouttefangeas, C., S. Jacquot, E. Meffre, M. Schmid, L. Boumsell, and A. Bensussan. 1994. Differential proliferative responses in subsets of human CD28⁺ cells delineated by BB27 mAb. *Int. Immunol. 6:423.*
28. Levine, B. L., Y. Ueda, N. Craighead, M. L. Huang, and C. H. June. 1995. CD28 ligands CD80 (B7-1) and CD86 (B7-2) induce long-term autocrine growth of CD4⁺ T cells and induce similar patterns of cytokine secretion in vitro. *Int. Immunol. 7:891.*
29. Weng, N. P., B. L. Levine, C. H. June, and R. J. Hodes. 1995. Human naive and memory T lymphocytes differ in telomeric length and replicative potential. *Proc. Natl. Acad. Sci. USA 92:11091.*
30. Connors, M., J. A. Kovacs, S. Krevat, J. C. Gea-Banacloche, M. C. Sneller, M. Flanigan, J. A. Metcalf, R. E. Walker, J. Falloon, M. Baseler, R. Stevens, I. Feuerstein, H. Masur, and H. C. Lane. 1997. HIV infection induces changes in CD4⁺ T-cell phenotype and depletions within the CD4⁺ T-cell repertoire that are not immediately restored by antiviral or immune-based therapies. *Nat. Med. 3: 533.*
31. Lindsten, T., C. H. June, J. A. Ledbetter, G. Stella, and C. B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science 244:339.*
32. McHugh, S., J. Deighton, I. Rifkin, and P. Ewan. 1996. Kinetics and functional implications of Th1 and Th2 cytokine production following activation of peripheral blood mononuclear cells in primary culture. *Eur. J. Immunol. 26:1260.*
33. Romagnani, S. 1995. Biology of human TH1 and TH2 cells. *J. Clin. Immunol. 15:121.*
34. King, C. L., R. J. Stupi, N. Craighead, C. H. June, and G. Thyphronitis. 1995. CD28 stimulates IL-4 and IL-5 production and promotes Th_2 subset differentiation by human CD4⁺ cells. *Eur. J. Immunol. 25:587.*

35. Riddell, S. R., and P. D. Greenberg. 1990. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. *J. Immunol. Methods 128:189.*
36. Sunder-Plassman, R., H. Breitneder, K. Zimmermann, D. Strunk, O. Majdic, W. Knapp, and W. Holter. 1996. Single human T cells stimulated in the absence of feeder cells transcribe interleukin-2 and undergo long-term clonal growth in response to defined monoclonal antibodies and cytokine stimulation. *Blood 87: 5179.*
37. Costello, R., C. Cerdan, C. Pavon, H. Brailly, C. Hurpin, C. Mawas, and D. Olive. 1993. The CD2 and CD28 adhesion molecules induce long-term autocrine proliferation of CD4$^+$ T cells. *Eur. J. Immunol. 23:608.*
38. Effros, R. B., N. Boucher, V. Porter, X. Zhu, C. Spaulding, R. L. Walford, M. Kronenberg, D. Cohen, and F. Schachter. 1994. Decline in CD28$^+$ T cells in centenarians and in long-term T cell cultures: a possible cause for both in vivo and in vitro immunosenescence. *Exp. Gerontol. 29:601.*
39. Caruso, A., A. Cantalamessa, S. Licenziati, L. Peroni, E. Prati, F. Martinelli, A. D. Canaris, S. Folghera, R. Gorla, A. Balsari, R. Cattaneo, and A. Turano. 1994. Expression of CD28 on CD8$^+$ and CD4$^+$ lymphocytes during HIV infection. *Scand. J. Immunol. 40:485.*
40. Borthwick, N. J., M. Bofill, W. M. Gombert, A. N. Akbar, E. Medina, K. Sagawa, M. C. Lipman, M. A. Johnson, and G. Janossy. 1994. Lymphocyte activation in HIV1 infection. II. Functional defects of CD28T cells. *AIDS 8:431.*
41. Choremi-Papadopoulou, H., V. Viglis, P. Gargalianos, T. Kordossis, A. Iniotaki-Theodoraki, and J. Kosmidis. 1994. Downregulation of CD28 surface antigen on CD4$^+$ and CD8$^+$ T lymphocytes during HIV-1 infection. *J. Acquired Immune Defic. Syndr. 7:245.*
42. Lewis, D. E., D. S. Tang, A. Adu-Oppong, W. Schober, and J. R. Rodgers. 1994. Anergy and apoptosis in CD8$^+$ T cells from HIV-infected persons. *J. Immunol. 153:412.*
43. Lake, R. A., R. E. O'Hehir, A. Verhoef, and J. R. Lamb. 1993. CD28 mRNA rapidly decays when activated T cells are functionally anergized with specific peptide. *Int. Immunol. 5:461.*
44. Lloyd, T. E., L. Yang, D. Ng Tang, T. Bennett, W. Schober, and D. E. Lewis. 1997. Regulation of CD28 costimulation in human CD8$^+$ T cells. *J. Immunol. 158:1551.*
45. Geppert, T. D., and P. E. Lipsky. 1988. Activation of T lymphocytes by immobilized monoclonal antibodies to CD3: regulatory influences of monoclonal antibodies to additional T cell surface determinants. *J. Clin. Invest. 81:1497.*
46. Lenardo, M. J. 1991. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature 353:858.*
47. Kearney, E. R., T. L. Walunas, R. W. Karr, P. A. Morton, D. Y. Loh, J. A. Bluestone, and M. K. Jenkins. 1995. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4$^+$ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J. Immunol. 155:1032.*
48. Smith, K. A., S. Gillis, P. E. Baker, D. McKenzie, and F. W. Ruscetti. 1979. T-cell growth factor-mediated T-cell proliferation. *Ann. NY Acad. Sci. 332:423.*
49. Gullberg, M. and K. A. Smith. 1986. Regulation of T cell autocrine growth: T4+ cells become refractory to interleukin 2. *J. Exp. Med. 163:270.*
50. Perillo, N. L., R. L. Walford, M. A. Newman, and R. B. Effros. 1989. Human T lymphocytes possess a limited in vitro life span. *Exp. Gerontol. 24:177.*
51. Sutkowski, N., M. L. Kuo, P. S. Amenta, J. P. Dougherty, and Y. Ron. 1995. A peripheral blood-derived monolayer supports long-term cultures of human CD4$^+$ and CD8$^+$ T lymphocytes. *Blood 85:3213.*
52. Adibzadeh, M., H. Pohla, A. Rehbein, and G. Pawelec. 1995. Long-term culture of monoclonal human T lymphocytes: models for immunosenescence? *Mech. Ageing Dev. 83:171.*
53. Adibzadeh, M., E. Mariani, C. Bartoloni, I. Beckman, G. Ligthart, E. Remarque, S. Shall, R. Solana, G. M. Taylor, Y. Barnett, and G. Pawelec. 1996. Lifespans of T lymphocytes. *Mech. Ageing Dev. 91:145.*
54. Weng, N. P., B. L. Levine, C. H. June, and R. J. Hodes. 1996. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J. Exp. Med. 183:2471.*
55. Constant, S. L., and K. Bottomly. 1997. Induction of Th1 and Th2 CD4$^+$ T cell responses: the alternative approaches. *Annu. Rev. Immunol. 15:297.*
56. Liu, Y., and C. A. Janeway, Jr. 1992. Cells that present both specific ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells. *Proc. Natl. Acad. Sci. USA 89:3845.*
57. Engwerda, C. R., B. S. Handwerger, and B. S. Fox. 1994. Aged T cells are hyporesponsive to costimulation mediated by CD28. *J. Immunol. 152:3740.*
58. Schwab, R., P. Szabo, J. S. Manavalan, M. E. Weksler, D. N. Posnett, C. Pannetier, P. Kourilsky, and J. Even. 1997. Expanded CD4$^+$ and CD8$^+$ T cell clones in elderly humans. *J. Immunol. 158:4493.*
59. Mackall, C. L., T. A. Fleisher, M. R. Brown, M. P. Andrich, C. C. Chen, I. M. Feuerstein, M. E. Horowitz, I. T. Magrath, A. T. Shad, S. M. Steinberg, L. H. Wexler, and R. E. Gress. 1995. Age, thymopoiesis, and CD4$^+$ T-lymphocyte regeneration after intensive chemotherapy. *N. Engl. J. Med. 332:143.*